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14. ABSTRACT

Heartwater is a tick-borne infectious disease caused by the rickettsial organism Cowdria ruminantium, currently Ehrlichia ruminantium. It poses an imminent threat to the Western Hemisphere, where it could cause mortality in cattle and other ruminant livestock in excess of 70%. It has been reported in the Caribbean; and its vector, Amblyomma sparsum, has been found on imported African spurred tortoises (Geochelone sulcata) and leopard tortoises (Geochelone pardalis) in southern Florida in the United States, leading to an importation ban on these reptiles. Symptoms have not been previously reported in reptiles. Here, we report peracute and acute deaths in African vipers imported from Africa through Florida. Signs included vomiting mucoid fluid, diarrhea, emaciation, convulsions, and death. Postmortem showed few gross lesions. The most consistent peracute and acute lesions were the pulmonary lesions and pericarditis with considerable bloody fluid in the pericardial sac (hydropericardium). These lesions strongly resembled the lesions of heartwater and a coccobacillus of less than 1-micron diameter was isolated in viper cell culture. The outbreak was brought to a halt by tick control and treatment of all exposed snakes with tetracycline. This isolation, tetracycline sensitivity, clinical signs, preliminary results with polymerase chain reaction of pCS20 ORF, and the viper preference of the disease may indicate a Cowdria-related attenuated species that has adapted to infect reptiles or an emerging new form of this group of microbes.

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Emerging Tick-Borne Disease in African Vipers Caused by a *Cowdria*-Like Organism

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ABSTRACT: Heartwater is a tick-borne infectious disease caused by the rickettsial organism *Cowdria ruminantium*, currently *Ehrlichia ruminantium*. It poses an imminent threat to the Western Hemisphere, where it could cause mortality in cattle and other ruminant livestock in excess of 70%. It has been reported in the Caribbean; and its vector, *Amblyomma sparsum*, has been found on imported African spurred tortoises (*Geochelone sulcata*) and leopard tortoises (*Geochelone pardalis*) in southern Florida in the United States, leading to an importation ban on these reptiles. Symptoms have not been previously reported in reptiles. Here, we report peracute and acute deaths in African vipers imported from Africa through Florida. Signs included vomiting mucoid fluid, diarrhea, emaciation, convulsions, and death. Postmortem showed few gross lesions. The most consistent peracute and acute lesions were the pulmonary lesions and pericarditis with considerable bloody fluid in the pericardial sac (hydropericardium). These lesions strongly resembled the lesions of heartwater and a coccobacillus of less than 1-micron diameter was isolated in viper cell culture. The outbreak was brought to a halt by tick control and treatment of all exposed snakes with tetracycline. This isolation, tetracycline sensitivity, clinical signs, preliminary results with polymerase chain reaction of pCS20 ORF, and the viper preference of the disease may indicate a Cowdria-related attenuated species that has adapted to infect reptiles or an emerging new form of this group of microbes.

KEYWORDS: Cowdria; Ehrlichia; viper; heartwater

INTRODUCTION

Heartwater is a tick-borne (*Amblyomma variegatum*, *Amblyomma hebraeum*, *Amblyomma lepidum*, *Amblyomma maculatum*, and other *Amblyomma*)

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infectious disease caused by the rickettsial organism formerly classified as the single member of the Genus Cowdria (*Cowdria ruminantium*), but now classified as *Ehrlichia ruminantium*.[1–3] *E. ruminantium* possesses a life cycle that closely resembles that of Chlamydia, with reticular bodies for intracellular reproduction and elementary (dense) bodies for infection of other cells.[2] Heartwater, or Cowdriosis, poses an imminent threat to the Western Hemisphere, where it could cause mortality in cattle and other ruminant livestock in excess of 70%.[4–7] It has been reported in the Caribbean (Islands of Antigua, Guadaloupe, Marie Galante, and perhaps even Cuba).[6–8] Its vector (*Amblyomma sparsum*), positive for Cowdria by pCS20 polymerase chain reaction (PCR) and DNA hybridization,[9] has been found on imported African spurred tortoises (*Geochelone sulcata*) and leopard tortoises (*Geochelone pardalis*) in southern Florida in the United States, leading to an importation and interstate movement ban on these reptiles.[10,11] Here, we report peracute and acute deaths in African vipers imported from Ghana (a major source of heartwater in West Africa),[12] and subsequent lethal secondary infections in cobras and United States' colubrid snakes, in a private collection, which closely resemble heartwater in signs and gross lesions. Culture, isolation, and molecular biology of the associated rickettsia are described.

MATERIALS AND METHODS

Clinical History and Ticks

On July 8, 2002, a juvenile Gaboon viper (*Bitis gabonica*) was presented dead as the first index case. From that time until February 19, 2003, a total of 22 snakes within the private collection showed similar neurological signs, including convulsions, followed by death. These snakes included eastern Gaboon vipers (*Bitis gabonica gabonica*), western Gaboon vipers (*Bitis gabonica rhinoceros*), rhinoceros vipers (*Bitis nasicornis*), a Sri Lankan cobra (*Naja naja polycellata*), a monocellate cobra (*Naja naja kaouthia*), a black-necked cobra (*Naja nigricollis*), and bullsnakes (*Pituophis melanoleucus sayi*). The outbreak only stopped after chemical removal of ticks with the topical pesticide permethrin (Provent-a-mite®, 0.5% permethrin; Pro Products, Mahopac, NY), and oral treatment of exposed snakes with tetracycline for 2 weeks. Other signs noted were swelling of the head and face, diarrhea, blindness, and vomiting of frothy mucoid material. Tissue samples from the lungs and liver were taken for standard sheep blood agar primary culture and isolation and some were retained for tissue culture in viper cells and bovine endothelial cells.

Two tick species were found on the premises (unfortunately collected following treatment with pesticide). These were the specific reptile tick *Aponomma*

latum, which has not been associated with heartwater transmission, and *A. maculatum*, which can transmit heartwater, but has not been reported to parasitize reptiles.[4,8]

Cell Lines, Bacterial Strains, and Culture

Russell's Viper snake spleen epithelial cells (ATCC CCL-129) and bovine pulmonary artery vascular endothelium endothelial cells (ATCC CRL-1733) were obtained from the American Type Culture Collection, Rockville, MD. Viper snake spleen epithelial cells were cultured at 30°C with 5% CO_2 in minimal essential medium (MEM eagles ATCC 30-2003) with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), and pen/strep (Sigma-Aldrich, St. Louis, MO). Liver homogenate was prepared from an isolated infected juvenile Gaboon viper snake liver. One hundred micrograms of the liver was homogenized in 500 μL MEM without FBS. Homogenate was transferred into a sterile microcentrifuge tube, an additional 500 μL of MEM was added, and the mixture was centrifuged at 7500 rpm for 15 min. The supernatant was filter sterilized (0.45 μm filter) and stored at −80°. Cell lines were infected with viper liver homogenate at 30°C with 5% CO_2 in MEM media and cytopathology was determined 24 h postexposure. Slide cultures of infected and mock-infected samples were processed for scanning electron microscopy (SEM).

Sterile swab samples were collected from the interior of lungs of one rhino viper and two eastern Gaboon vipers during postmortem examination. These were cultured on sheep blood agar and subcultured on liquid medium for biochemical analysis at Wilford Hall Medical Center, Lackland Air Force Base, Texas.

Microscopy

Upon receipt of cell cultures that still contained medium, the wells were filled with 3% glutaraldehyde, and the contents were fixed for 5 min at room temperature. The tip of a disposable pipette was placed in the very corner of each well and the medium/fixative mixture was slowly removed. Then, the wells were refilled with 3% glutaraldehyde and fixed for 1–2 h. After removing the glutaraldehyde from each well, the plastic wells and silicone gasket attached to the slide were gently peeled away. The slide wells were rinsed with distilled water and dehydrated with a graded series of ethanol. The slide was scored to separate the samples, mounted on aluminum specimen stubs, sputter-coated with a Hummer Sputter Coater (Hayward, CA), and viewed with a Philips XL20 SEM (Hillsboro, OR).

Primer Preparation and Molecular Biology

Three primers for pCS20 DNA were prepared with the following sequences:
AP126 GTAACACAATCTAAACTCGGTAAG[9]
AP127 CAGCCATACCTGACACGTATTCAT[9]
AP128 ACTAGTAGAAATTGCACAATCTAT[9]
AP129 TGATAACTTGGTGCGGGAAATCCTT[9]
pCS20F CTCACCCAAGTGTTCTTTC[10]
pCS20R GGTAACATTATATACAGCCATACCTGACAC

PCR was performed as described by Mahan *et al.*[9] Samples were run on 3% agarose gels in 1× TAE buffer. PCR products were precipitated using equal volume of 5 M ammonium acetate and two volumes of isopropanol. Samples were left at −20°C overnight. Samples were pooled and run on 3% gels. Bands were cut out and extracted from melted agarose by phenol extraction with lithium chloride and precipitation with ethanol. Precipitated DNA was dissolved in 5 μL DNA buffer and 1 μL run on an agarose gel to check size and concentration. Sequencing was performed.

RESULTS

Postmortem Gross Lesions

The postmortem of the juvenile Gaboon viper (index case) on July 8, 2002 revealed cheesy material in the trachea, a hyperemic lung, and kidneys with unusual red mottling. Acute pneumonia was the immediate cause of death. On August 7, 2002, a rhino viper died of pneumonia and shock based on the postmortem examination. On August 31, 2002, three more snakes were posted—a rhino viper adult (*B. nasicornis*), which had caseous mucoid material in the intestines and lung; a male adult eastern Gaboon (*Bitis gabonica gabonica*) viper with mucus in the esophagus, a hemorrhagic lung with caseous mucus, and a mottled liver; and a female adult eastern Gaboon viper with a hemorrhagic lung with fibrous and caseous mucoid material, large fat bodies, and the other organs essentially normal. On November 12, 2002, a Sri Lankan cobra (*Naja naja polycellata*, male) that died sometime in the last 3 days was found. It had lung hemorrhage with muco-purulent exudates, abundant peritoneal fluid, and dark tubules in kidneys delineated with urate crystals. At this time a juvenile Gaboon viper had died and was posted showing the same lung lesions as the other vipers and cobra. On February 19, 2003, a monocellate cobra (*Naja naja kaouthia*) died. At time of death, it was convulsive, had dry gangrene at tip of tail, and showed evidence of blindness. On February 23, 2003 it was posted, the cobra showed frothy deteriorated, bloody spots and necrosis on inner surface of lung, bloody lung and peritoneal fluid, focal white liver lesions throughout liver, enlarged pale heart muscle, blood that failed to clot in major vessels,

abundant fat bodies, bloody fluid coming from mouth, and kidneys dark and swollen with a "bunch of grapes" surface.

Culture, Isolation, and Microscopic Results

Material for routine bacterial culture from the rhino and Gaboon vipers (as described above) yielded a nonviable organism on subculture from the rhino and *Proteus retgerri*, *Klebsiella oxytoca*, *Flavobacterium* spp., *Streptococcus uberus*, *Enterococcus casseliflavus*, and *Staphylococcus sciuri* from primary and subcultures from the Gaboon vipers. The standard bacteriology was inconsistent, with the disease syndrome not being attributable to any specific free-living bacterial agent. This observation led to the co-culturing of liver tissue isolated from the last juvenile Gaboon viper to die with viper cells and bovine endothelial cells. FIGURE 1 shows the cytopathic effects (light microscopy) on viper cells following a week of culture and bovine endothelial cells after

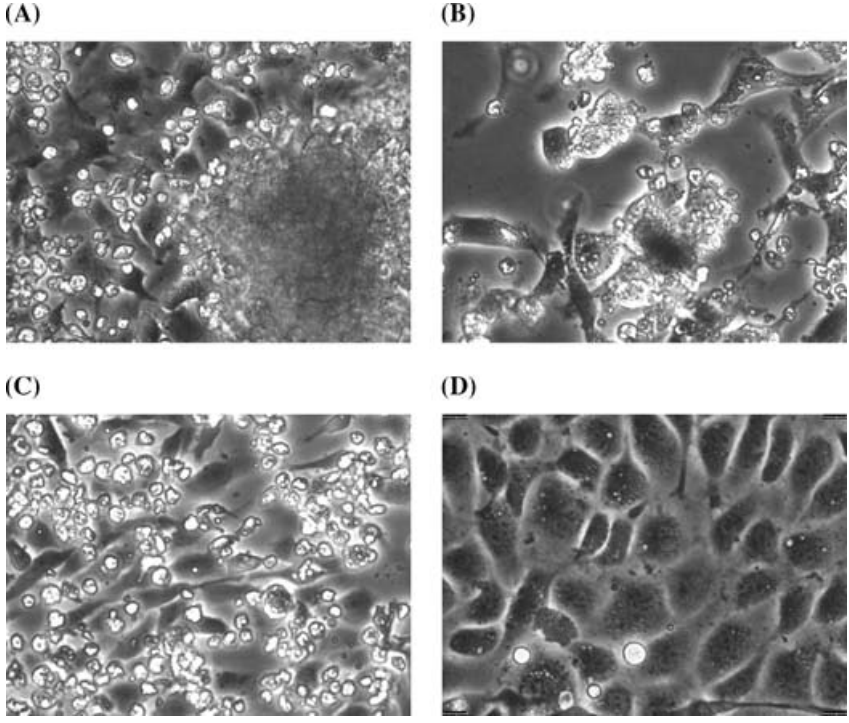

FIGURE 1. (A) Cytopathic effect (light microscopy) on viper cells following a week of culture with Cowdria-like microbes, and, **(B)**, bovine endothelial cells following 24 h of culture with Cowdria-like microbes. Both are compared with their respective controls (**C** and **D**).

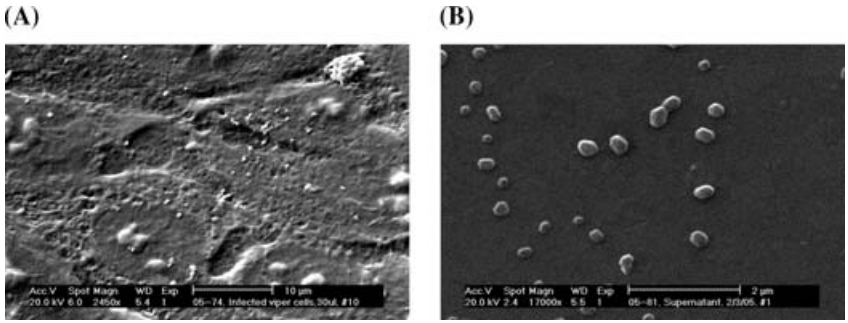

FIGURE 2. (A) SEMs of emerging clusters and individual microbes from the cytoplasm of viper cells. **(B)** Individual "elementary bodies," or dense bodies, of the microbe isolated from the viper cell cultures (approximately 200 nm in diameter).

24 h of infection with a concentrate from the viper cell culture. FIGURE 2 shows SEMs of emerging clusters and individual microbes from the cytoplasm of the viper cells and displays the individual "elementary bodies," or dense bodies, of the microbe isolated from the viper cell cultures (approximately 200 nm in diameter). FIGURE 3 shows the cellular lesions of the bovine endothelial cell culture at the single cell level.

PCR Results

FIGURE 4 shows PCR results from viper cells and original snake tissues including hepatic tissue from the juvenile Gaboon viper and a bullsnake and heart blood from adult rhino and Gaboon vipers. In many cases, PCR products were produced that matched in both the viper cells and postmortem tissue samples, but the multiple products were significantly different though related to those produced by authentic *E. ruminantium,* according to the literature.[1]

DISCUSSION

On December 21, 1999, the State of Florida Department of Agriculture and Consumer Services, Division of Animal Industry, issued a Florida Fish and Wildlife Conservation Emergency Prohibition against importation of certain African tortoises.[11,12] It banned the importation of African spurred tortoises (*G. sulcata*) and leopard tortoises (*G. pardalis*). The emergency ban was due to the finding that some tortoises were infected with "tropical bont ticks" carrying the rickettsia *E. ruminantium,* causative agent of heartwater. Ticks collected from a Hillsborough County, Florida, reptile facility tested positive for heartwater agent on November 29, 1999. On December 9, 1999, the Florida

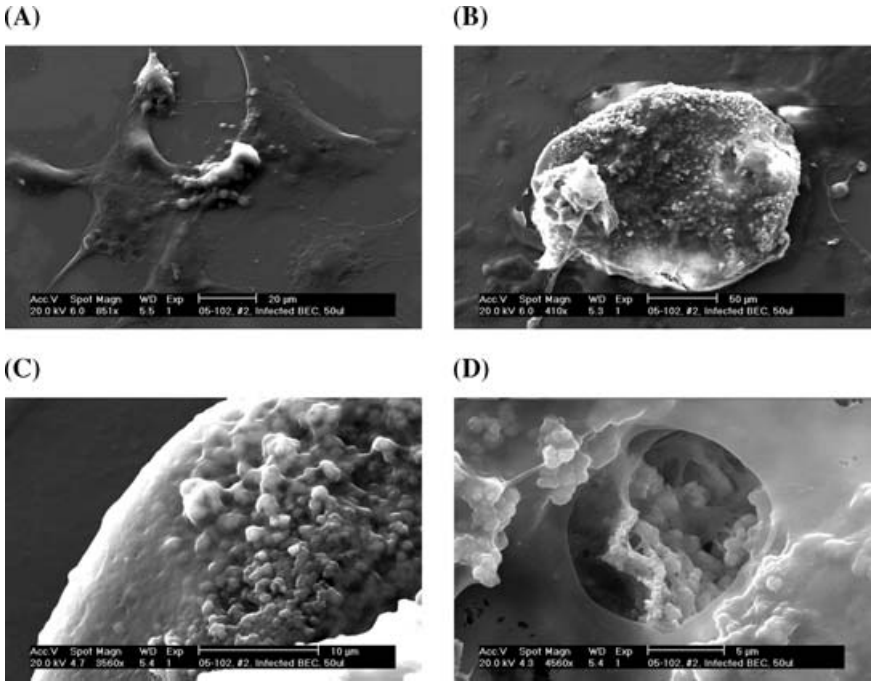

FIGURE 3. (**A–D**) The cellular lesions of the bovine endothelial cell culture at the single cell level (SEM).

Department of Agriculture and Consumer Services passed an emergency rule restricting the importation into the State of all wildlife without an official Certificate of Veterinary Inspection showing the animals to be free of heartwater and its vectors. The data presented here raises question of whether the rule can be enforced and whether sufficient technology exists to execute the order effectively. Snakes are not routinely examined for the disease or its vectors, in spite of many being imported through Miami, Florida, primarily from a high heartwater incidence area in Africa, Ghana.[13] The snakes reported here showing signs that strongly resemble those of heartwater in ruminants have been reported in the past to carry the vectors of heartwater.[4] The quandary exists, based on the preliminary results described herein, as to whether snakes are reservoirs (carriers) of true heartwater, or of some other disease that closely resembles it or is derived from it (through mutation, attenuation, and adaptation to snakes as hosts and their ectoparasites as vectors), that should be controlled. The successful infection of bovine endothelial cells strongly suggests the first case. What is clear is that the viper disease is highly pathogenic for snakes, crosses over to many species of snakes, and poses a threat to any zoo or private collection into which infected snakes enter. It could also enter native wildlife

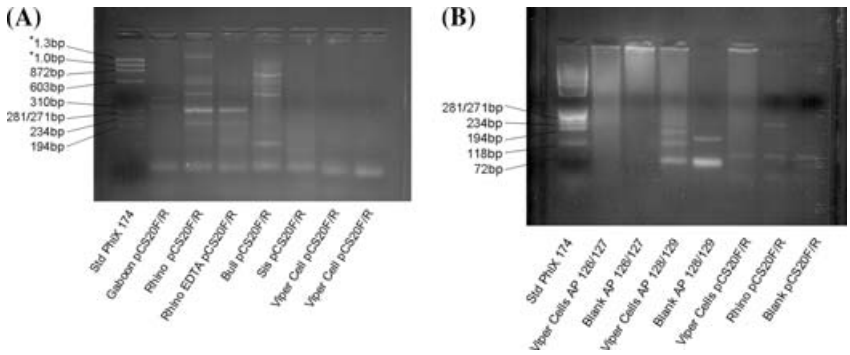

FIGURE 4. (**A, B**) Electrophoresis gel of PCR products of pCS20 primers interacting with DNA from viper cell cultures and from original snake tissues including hepatic tissue from the juvenile Gaboon viper and a bullsnake, and heart blood from adult rhino viper, Sis = *Sistrurus catenatus edwardsi*, Desert Massasauga rattlesnake, control. *First two bands in panel A are kilobase pairs.

populations of snakes, other reptiles, birds, and mammals. Although control of the tick vectors may be adequate, other blood-feeding arthropods (such as mites) could spread the disease from chronically infected snakes, or this rickettsia may be sufficiently different from *E. ruminantium* to switch to a direct transmission life cycle. At a minimum, the viper disease presents "diagnostic confusion" for the veterinary and regulatory officials who are trying to keep heartwater out of the continental United States. In honor of the discoverer of the disease, acknowledgment of its origin in the snake genus *Bitis*, and its place of origin, Ghana, we recommend, that if it is a new species, it be called *Ehrlichia bishopii v. bitighanae*, or if it proves to be a strain of *E. ruminantium*, that it be called *E. ruminantium v. bishopii*.

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